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Nutrient Sensor in the Brain Directs the Action of the Brain-Gut Axis in *Drosophila*

Highlights

- Six Dh44+ cells in the brain are essential for post-ingestive nutrient selection
- Dh44 neurons are specifically activated by nutritive sugars found in the hemolymph
- Activation of the Dh44 circuit results in increased proboscis extension and excretion
- The brain-gut axis connects the ability to detect nutrients with effector mechanisms

Authors

Monica Dus, Jason Sih-Yu Lai, Keith M. Gunapala, ..., Eliot Geraud, Christina M. Joseph, Greg S.B. Suh

Correspondence

greg.suh@med.nyu.edu

In Brief

Sugar is sweet and nutritive. The taste cells that detect its sweetness were identified, but the sensor that detects its nutritional value is unknown. Dus et al. identify a brain-gut microcircuit expressing Dh44/CRH as the nutrient sensor.

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Nutrient Sensor in the Brain Directs the Action of the Brain-Gut Axis in *Drosophila*

Monica Dus,[1,3] Jason Sih-Yu Lai,[1] Keith M. Gunapala,[1] Soohong Min,[1] Timothy D. Tayler,[2] Anne C. Hergarden,[2] Eliot Geraud,[1] Christina M. Joseph,[1] and Greg S.B. Suh[1,*]
[1]Department of Cell Biology, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA
[2]Division of Biology 156-29, California Institute of Technology, 1200 E. California Boulevard, Pasadena, CA 91125, USA
[3]Present address: Department of Molecular, Cellular, and Developmental Biology, The University of Michigan, 830 North University Avenue, Ann Arbor, MI 48109, USA
*Correspondence: greg.suh@med.nyu.edu
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SUMMARY

Animals can detect and consume nutritive sugars without the influence of taste. However, the identity of the taste-independent nutrient sensor and the mechanism by which animals respond to the nutritional value of sugar are unclear. Here, we report that six neurosecretory cells in the *Drosophila* brain that produce Diuretic hormone 44 (Dh44), a homolog of the mammalian corticotropin-releasing hormone (CRH), were specifically activated by nutritive sugars. Flies in which the activity of these neurons or the expression of *Dh44* was disrupted failed to select nutritive sugars. Manipulation of the function of Dh44 receptors had a similar effect. Notably, artificial activation of Dh44 receptor-1 neurons resulted in proboscis extensions and frequent episodes of excretion. Conversely, reduced Dh44 activity led to decreased excretion. Together, these actions facilitate ingestion and digestion of nutritive foods. We propose that the Dh44 system directs the detection and consumption of nutritive sugars through a positive feedback loop.

INTRODUCTION

Sugars in the natural environment can be detected through taste-dependent and taste-independent modalities. Taste-dependent modalities consist mainly of peripheral taste receptor cells such as sweet-sensing cells, which primarily detect the palatability of sugar (for a review, see Yarmolinsky et al., 2009) Evidence of a taste-independent modality was shown more than 20 years ago when investigators showed that rodents could learn to select a flavored solution when it was paired with an intragastric infusion of nutritive sugars but not with water or nonnutritive saccharin (for a review, see Sclafani and Ackroff, 2012). This finding was further demonstrated by experiments using taste-insensitive *Trpm5* (−/−) mice, which learn to associate nutritive sugars paired with a conditioned stimulus independent of taste input (de Araujo et al., 2008). Similarly, fruit flies—*Drosophila melanogaster*—are capable of associating the caloric value of sugars with an odorant to establish a long-term memory (Burke and Waddell, 2011; Fujita and Tanimura, 2011; Musso et al., 2015).

While animals and humans can learn to recognize the nutritional value of sugar during sugar-preference conditioning (Birch et al., 1990; Brunstrom and Mitchell, 2007; Yeomans et al., 2008), *Drosophila* do not need to be trained to distinguish between nutritive sugars and nonnutritive sugars. Studies have shown that naive flies that had not previously been exposed to nutritive sugars or nonnutritive sugars were still able to select nutritive sugars over nonnutritive ones after periods of food deprivation in a two-choice preference assay (Dus et al., 2011; Miyamoto et al., 2012; Stafford et al., 2012). The post-ingestive preference for a nutritive sugar appears to be mediated by a hardwired neuronal pathway that is activated by the detection of nutritive sugars. However, the molecular and cellular identity of the nutrient sensor and the neural circuitry that allows flies (as well as mammals) to respond to the nutritional value of exogenous sugar is largely unknown.

The postprandial increase in the intestinal and circulating glucose levels plays an important role in the ability of animals to choose conditioned stimuli paired with nutritive sugars. Several studies in rodents showed that intravenous glucose administration is sufficient for preference conditioning, while direct stimulation of the intestinal mucosa was also shown to be important (Mather et al., 1978; Oliveira-Maia et al., 2011; Tordoff and Friedman, 1986; Zukerman et al., 2013). This relationship was further supported by the observation in flies that administrating phlorizin, which lowers hemolymph glycemia by inhibiting sugar transport, blocked the flies' ability to select nutritive sugars (Dus et al., 2013). Notably, taste-independent sugar conditioning was shown to correlate with the rate of glucose utilization instead of circulating glucose levels in mice (Ren et al., 2010). In humans, the physiological parameter that appears to correlate with preference conditioning is also metabolic responses to glucose (de Araujo et al., 2013). While these studies illustrate that utilizing intracellular glucose is crucial for activating

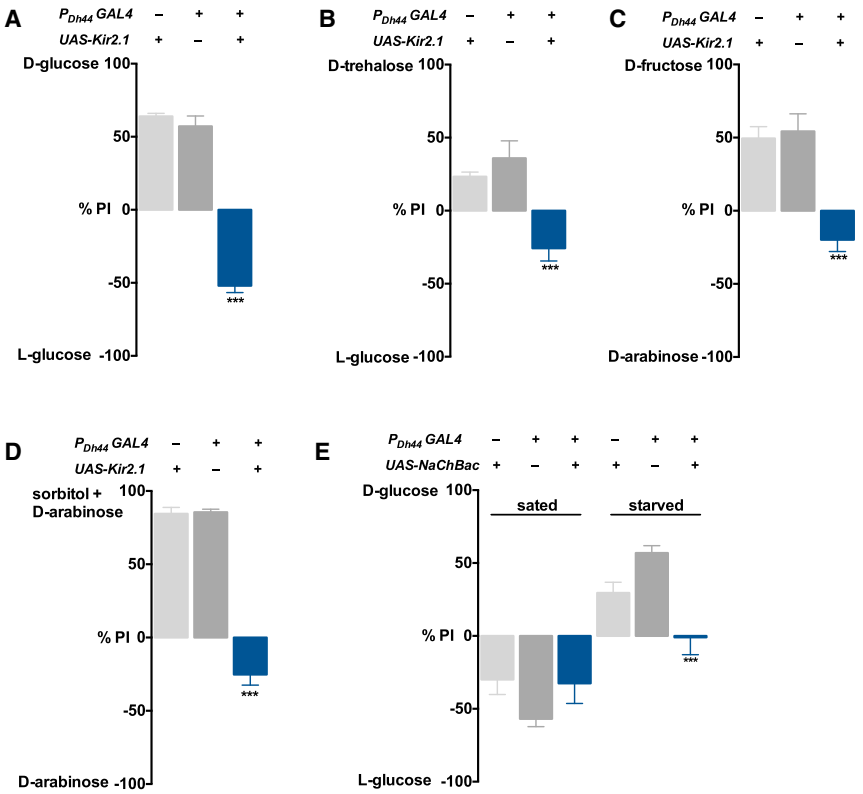

Figure 1. Manipulating the Activity of Dh44 Neurons Perturbs Post-ingestive Nutrient Selection

The food preference of flies that were given a choice between a sweeter yet nonnutritive sugar (L-glucose or D-arabinose) and a nutritive sugar (D-glucose, D-trehalose, D-fructose, or sorbitol) after 5-hr (sated) or 18-hr (starved) food deprivation. (A–D) Inactivation of Dh44 neurons by expression of the *UAS-Kir2.1* transgene using the P_{Dh44}-GAL4 driver (blue bars) abolishes preference for nutritive sugars in starved flies. Flies carrying each transgene alone were used as controls (gray bars). Flies were given a choice: (A) 200 mM L-glucose versus 50 mM D-glucose; (B) 200 mM L-glucose versus 100 mM D-trehalose; (C) 80 mM D-arabinose versus 25 mM D-fructose; and (D) 20 mM D-arabinose versus 20 mM D-arabinose + 80 mM sorbitol. Each shade of gray corresponds to one control.

(E) Artificial activation of Dh44 neurons by expression of *UAS-NaChBac* using P_{Dh44}-GAL4 abolishes the preference for D-glucose over L-glucose in starved flies but does not affect sated flies. Flies bearing each transgene alone were used as controls. n = 4–10, with each trial comprising approximately 40 flies for this and all subsequent behavior figures.

***p < 0.001 (one-way ANOVA with Tukey post hoc test). Error bars indicate SEM.

behavioral responses, circulating plasma glucose level is key in determining intracellular glucose concentration.

Indeed, Jean Mayer proposed over 5 decades ago that feeding is regulated by neurons in the brain that sense circulating blood glucose levels (Mayer, 1953). This ''glucostatic hypothesis'' was substantiated by the discovery of glucose-sensing neurons in the hypothalamus (Anand et al., 1964; Oomura et al., 1964). These specialized neurons use the products of glucose metabolism to regulate neuronal excitability and neurotransmitter release. Metabolic enzymes such as glucokinase, the AMP-activated protein kinase (AMPK), and the ATP-sensitive K^+ (K_{ATP}) channel were implicated in mediating this process (Kang et al., 2004; Minokoshi et al., 2004). However, the disruption of K_{ATP} channel or AMPK function in glucose-excited pro-opiomelanocortin (POMC) neurons, which impaired their ability to sense glucose, did not result in a discernable feeding phenotype in mice (Claret et al., 2007; Parton et al., 2007). While several populations of glucose-sensing neurons have been identified in the hindbrain and hypothalamus, their biological role in feeding-related behavior is still elusive (Levin, 2007).

In this work, we identified a population of neurons in the fly brain producing the Diuretic hormone 44 neuropeptide (Dh44, the insect homolog of the mammalian CRH) (Lovejoy and Jahan, 2006) that is essential for mediating taste-independent behavioral responses to the nutritional value of sugar. Calcium imaging revealed that Dh44 neurons are activated by solutions containing nutritive sugars and require a functional glucokinase enzyme to detect these sugars. The Dh44 neuropeptide conveys the information from Dh44 neurons to Dh44 receptor R1 neurons in the brain and R2 cells in the gut, both of which are also required for nutrient selection. Furthermore, artificial activation of Dh44 R1 neurons stimulated rapid proboscis extension reflex (PER) responses, promoting food intake. Flies with activated Dh44 R1 neurons also excreted more frequently, a behavior likely increased by gut motility. Conversely, reduced Dh44 signaling resulted in a lower frequency of excretion. We propose that this putative post-ingestive nutrient sensor activates two pathways: one to promote PER to reinforce the ingestion of nutritive foods and another to enhance the gut motility, which would facilitate digestion of greater volumes of the nutritive foods.

RESULTS

The Activity of Dh44 Neurons Is Essential for Post-ingestive Nutrient Selection

To identify the neural circuitry that underlies the post-ingestive effects of nutritive sugars, we searched for neurons that are required for selection of a nutritive sugar over a nonnutritive sugar after periods of food deprivation. We screened a collection of neuropeptide GAL4 lines crossed to *UAS-Tetanus toxin* (*UAS-TNT*), which eliminates synaptic transmission, in the two-choice assay. Nearly all of these tested fly lines chose a more concentrated, yet nonnutritive L-glucose (200 mM) when they were sated, but developed a preference for nutritive D-glucose (50 mM) when they were starved for 18 hr. We found that inactivation of Dh44 neurons by expressing Kir2.1, an inwardly rectifying K^+ channel (Nitabach et al., 2002), abolished the preference for D-glucose in starved flies (Figure 1A). We investigated the

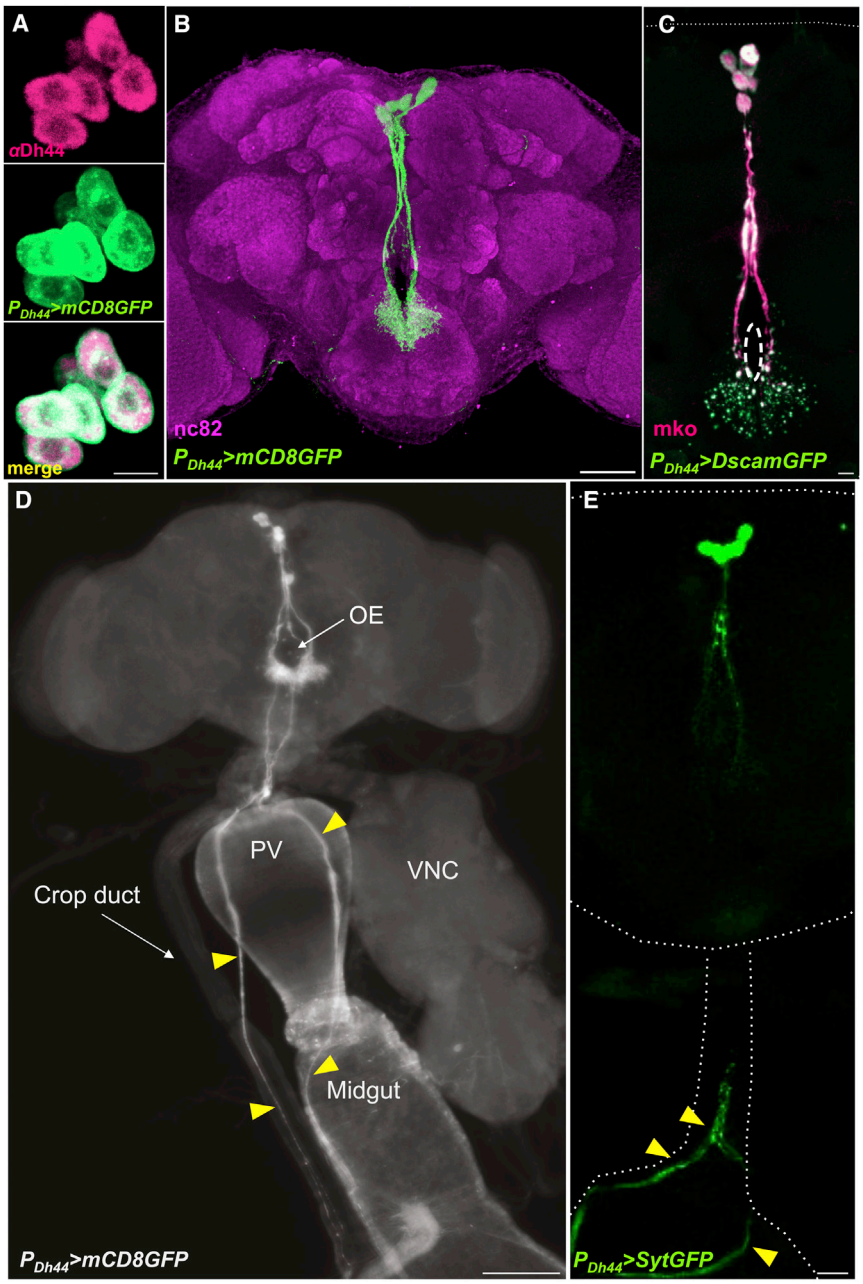

Figure 2. The Expression Pattern of the P_{Dh44}-GAL4 Line

(A) The reporter P_{Dh44}-*GAL4* > *mCD8GFP* (green) labels Dh44 cells in the PI, visualized by anti-Dh44 antibody (pink) in a z-stack confocal image with 1-μm optical sections. Merge is in yellow. Scale bar, 10 μm.

(B) A z-stack image of the brain of a fly carrying P_{Dh44}-GAL4 and *UAS-mCD8GFP* (green) counterstained with the neuropil marker nc82 (magenta). Scale bar, 50 μm.

(C) The dendritic arborization of Dh44 neurons, visualized by P_{Dh44}-*GAL4* > *DscamGFP* (green), in the dorsal region of the SEZ. Dh44 cell bodies and processes are labeled by a fluorescent marker, Monomeric Kusabira Orange (mko) (pink). Dotted circle depicts the esophagus. Scale bar, 10 μm.

(D) The neurites of P_{Dh44}-*GAL4* > *mCD8GFP* cells innervate the gut and crop (yellow arrowheads). OE, esophagus; PV, proventriculus; VNC, ventral nerve cord. Scale bar, 100 μm.

(E) The axons of Dh44 neurons, visualized by P_{Dh44}-*GAL4* > *SytGFP* (green), descend along the esophagus to innervate the gut (yellow arrowheads) revealed in a z-stack image. Scale bar, 20 μm.

possibility that Dh44 neurons are also required for flies to select two other sugars that are normally present in the hemolymph: D-trehalose, which, like D-glucose, is found in abundance and D-fructose, which is found in a minute amount (Miyamoto et al., 2012). We gave flies carrying P_{Dh44}-GAL4 and *UAS-Kir2.1* a choice between these nutritive sugars and nonnutritive sweeteners. These flies failed to select D-trehalose or D-fructose over higher concentrations of the nonnutritive sweeteners (Figures 1B and 1C). They also failed to respond to the tasteless yet nutritive sorbitol (Figure 1D). These observations indicate that Dh44 neurons play an important role in mediating the selection of nutritive sugars independent of taste input.

We then examined whether artificial activation of Dh44 neurons is sufficient to communicate the reward of nutrient, even when nonnutritive sugars are fed. For this experiment, we generated flies expressing NachBac (Nitabach et al., 2005), a bacterial sodium channel that increases the electrical excitability of neurons under the control of P_{Dh44}-GAL4, and tested them in the two-choice assay (D- versus L-glucose). These flies did not demonstrate a preference for the nutritive sugar, even though they had not been fed for 18 hr (Figure 1E). Instead, they consumed both D- and L-glucose indiscriminately, likely because the both glucose enantiomers are perceived as nutritious to these flies when Dh44 neurons are artificially activated. Thus, Dh44 neurons are necessary and sufficient for post-ingestive nutrient sensing behavior. Inactivation or activation of Dh44 neurons, however, did not affect the amount of food intake (Figure S1), suggesting that these neurons selectively control food choice behavior instead of food consumption.

Six Dh44 Neurons Localized to the *pars intercerebralis* Mediate the Behavior

To determine the expression pattern of the P_{Dh44}-GAL4 line, we crossed it to *UAS-mCD8GFP* and found six labeled cells located in the *pars intercerebralis* (PI) (Figure 2), a region of the fly brain populated with neurosecretory cells. It has been suggested

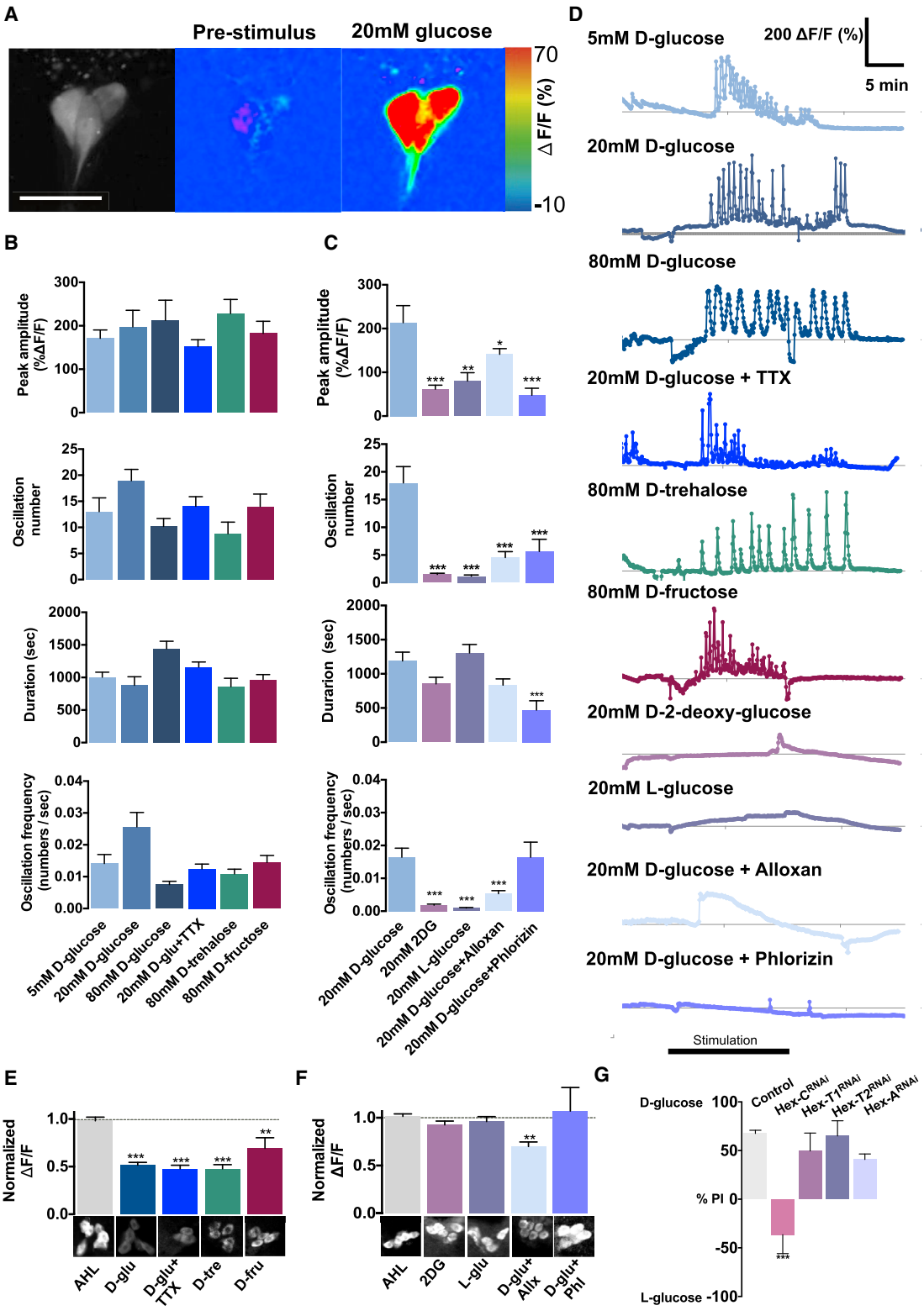

Figure 3. Activation of Dh44 Neurons by Nutritive Sugars Promotes the Secretion of Dh44 Neuropeptide

(A–D) The ex vivo brain preparations of flies carrying P_{Dh44}-GAL4 and *UAS-GCaMP3.0* were exposed to AHL saline containing different sugars.

(A) The response (ΔF) of Dh44 neurons to AHL containing 20 mM D-glucose (right). Pre-stimulation images show the position of six Dh44 cells (left) and the response to the control AHL containing 20 mM sucrose (middle). Scale bar, 20 μm.

(legend continued on next page)

that the PI serves as the functional correlate of the mammalian hypothalamus (de Velasco et al., 2007). The six labeled cells were indeed immunopositive for anti-Dh44 antibody (Zitnan et al., 1993) (Figure 2A). Given that the targeted expression of *Dh44* in these cells rescued the behavioral defects caused by the *Dh44* mutation (Figure 4B), these neurons are important for mediating the selection of nutritive sugars. Dh44 neurons project their neurites to the dorsal region of the subesophageal zone (SEZ) and also extend their lengthy processes along the esophagus to innervate the crop and midgut in the abdomen (Figures 2B and 2D). Using a fluorescent postsynaptic marker, Down Syndrome Cell Adhesion Molecule (Dscam)-GFP (Wang et al., 2004), we traced the dendrites of Dh44 neurons as they arborized in the SEZ (Figure 2C). Conversely, a presynaptic marker, synaptotagmin (Syt)-GFP (Zhang et al., 2002), expressed by the P_{Dh44}-GAL4 driver, illustrated that these neurons extend their axonal projections along the esophagus to innervate the gut (Figure 2E). In addition to these Dh44 cells located in the brain, three to four Dh44 neurons were found in the posterior ventral nerve cord (VNC) (data not shown).

Dh44 Neurons Are Activated by Nutritive Sugars and Not by Nonnutritive Sugars

Next, we asked whether Dh44 neurons respond to nutritive sugars. By performing calcium imaging on ex vivo brain preparations of flies carrying the fluorescent calcium indicator *UAS-GCaMP3.0* and P_{Dh44}-GAL4, we found that Dh44 neurons were activated by nutritive D-glucose, D-trehalose, and D-fructose with substantial calcium oscillations (Figures 3A–3D; Movie S1). In vivo calcium imaging also showed that Dh44 neurons respond to solutions containing nutritive sugar (Figure S2) (Ai et al., 2010). Sucrose, which was used in the control saline, did not stimulate these neurons (Figure S3), suggesting that only hemolymph sugars are effective. Calcium oscillations are a characteristic of neurosecretory cells and occur when these cells are secreting neuropeptides (Thorner et al., 1988). As the concentration of perfused nutritive sugars was increased, the frequency of these oscillations decreased, yet they were longer in duration. The oscillations were still clearly observed in response to D-glucose concentrations as low as 5 mM and approximately 4 min after the brain tissue was exposed to this sugar solution. By contrast, exposures to nonnutritive sugars L-glucose and 2-deoxy-glucose resulted in a very limited increase in calcium transit and completely lacked calcium oscillations (Figures 3C and 3D).

To determine whether sugar-induced activation of Dh44 neurons results in the release of Dh44 neuropeptide from these neuronal cells, we incubated *Drosophila* brains in saline solutions containing different sugars and then probed each brain by using anti-Dh44 antibody to measure the amount of Dh44 left inside the cells. We found significantly less Dh44 immunoreactivity in Dh44 cells of the brains exposed to nutritive sugars compared to those exposed to the control saline (Figure 3E). Among the nutritive sugars, D-glucose and D-trehalose had stronger effects than D-fructose. In contrast, exposures to the nonnutritive sugars L-glucose and 2-deoxy-glucose had no effect on Dh44 immunoreactivity (Figure 3F). These findings suggest that sugar-induced activation promotes the release of Dh44 neuropeptide from these neurons.

Consistent with these findings, inactivating Dh44 neurons by *UAS-Kir2.1* expression, which resulted in impaired post-ingestive nutrient selection, suppressed the secretion of Dh44 peptide, even when the Dh44 cells were stimulated by D-glucose (Figure S4). By contrast, artificial activation of Dh44 neurons by expressing of *UAS-NachBac*, which was sufficient to communicate the reward of nutrient, released the Dh44 peptide without sugar stimulation (Figure S4). Therefore, manipulating Dh44 neuronal function has distinct effects on the neuronal activity and peptide release, supporting the view that Dh44 neuropeptide is the signal that communicates the information about the rising levels of nutritive sugar in the internal milieu.

Dh44 Neuronal Responses Require Sugar Entry and a Hexokinase

The observation that Dh44 neurons respond specifically to nutritive sugars led us to consider the possibility that intracellular metabolism of nutritive sugars in these cells stimulated the release of Dh44 peptide. The first steps critical for glucose metabolism are the entry of glucose into the Dh44 cells and the conversion of glucose to glucose-6-phosphate by hexokinase. The sugar entry is required for the activation of Dh44 neurons, as an addition of phlorizin, an inhibitor of sugar transporters, in saline significantly reduced glucose-induced calcium oscillations and secretion of the Dh44 peptide (Figures 3C, 3D, and 3F). To determine whether a hexokinase is important for the activation of Dh44 neurons, we used alloxan, a well-known inhibitor of

(B) Quantification of Dh44 neuronal responses to nutritive sugars: D-glucose (5, 20, and 80 mM; blue bars), D-trehalose (green bars), and D-fructose (magenta bars), and D-glucose mixed with TTX (0.5 μM) (bright blue bars). Peak amplitude (ΔF/F) was obtained by subtracting the pre-stimulation baseline (average of 10–15 frames) from the sugar-evoked peak value. Oscillation number refers to the total number of calcium transients during stimulation. Duration is the length of Dh44 neuronal response to stimulation. Oscillation frequency is calculated as a ratio of oscillation number/duration. n = 9–27 cells.

(C) Quantification of Dh44 neuronal responses to nonnutritive sugars: 2-deoxy-D-glucose (2DG, lavender bar) and L-glucose (light purple bar), and D-glucose mixed with alloxan (4 μM), a hexokinase inhibitor (light blue bar), and D-glucose mixed with phlorizin (1 μM), a glucose transporter inhibitor (purple bar). n = 5–19 cells. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(D) Representative traces of Dh44 neuronal responses to different sugars.

(E and F) The immunofluorescence measurement of intracellular Dh44 neuropeptide, probed with anti-Dh44 antibody upon (E) stimulation of Dh44 cells with 80 mM D-glucose, D-trehalose, D-fructose, D-glucose mixed with TTX (0.5 μM), and the control AHL containing 80 mM sucrose (n = 24–42 cells); or (F) stimulation of Dh44 cells with 80 mM 2-deoxy-glucose, L-glucose, and D-glucose mixed with alloxan (Allx) or with phlorizin (Phl) (n = 22–43 cells). Representative images of Dh44 neurons stimulated by different sugars are shown below. **$p < 0.01$. ***$p < 0.001$.

(G) The food preference of flies carrying P_{Dh44}-GAL4 and *UAS-RNAi* for each hexokinase in the two-choice assay (50 mM D-glucose versus 200 mM L-glucose) after 18-hr starvation. Flies bearing P_{Dh44}-GAL4 alone were used as a control. n = 4–6. ***$p < 0.001$ (one-way ANOVA with Tukey post hoc test).

Error bars indicate SEM.

hexokinase (Lenzen et al., 1988), to block glucose metabolism. When Dh44 neurons were stimulated with a mixture of D-glucose and alloxan, calcium oscillations essentially disappeared (Figures 3C and 3D). Consistent with this, glucose-induced secretion of Dh44 from these cells was reduced in fly brains incubated with the mixture of glucose and alloxan compared to glucose alone (Figures 3E and 3F). Furthermore, *hexokinase C* (*Hex-C*), one of five hexokinases present in the *Drosophila* genome, is required for Dh44 neuronal activation, since RNAi-induced knockdown of *Hex-C* expression in these neurons led to a failure in responding to nutritive sugars in the two-choice assay (Figure 3G); *Hex-C* RNAi knockdown did not appear to cause anomaly, as the morphology of these neurons was indistinguishable from that of wild-type Dh44 neurons (Figure S5). Intriguingly, unlike other hexokinases, *Hex-C* is selectively expressed in the brain, fat body, and gut of the fly (Gelbart and Emmert, 2013). Pyruvate, the end product of the glycolysis pathway, also activated Dh44 neurons, further supporting a role of this metabolic pathway in the neuronal activation (Figure S6).

The fact that this pathway is autonomously required in Dh44 neurons supports the hypothesis that the function of activated Dh44 neurons is to facilitate the detection of nutritive sugars through direct activation. This hypothesis is further supported by our observation that an addition of tetrodotoxin (TTX, which blocks voltage-gated Na^+ channels and eliminates synaptic transmissions and indirect presynaptic responses) had virtually no effect on the release of Dh44 from Dh44 neurons (Figure 3E) and only an insignificant effect on their neuronal activity in response to D-glucose (Figure 3B).

Dh44, *Dh44* Receptors, and Dh44 Receptor Cells Mediate Post-ingestive Nutrient Selection

Having shown that Dh44 neurons mediate the secretion of Dh44 upon stimulation by nutritive sugars, we next determined whether the *Dh44* gene, the fly homolog of the human CRH (Figure 4A), is required for starved flies to select nutritive sugars during the two-choice assay. We found that *Dh44* mutants showed defects in their ability to select D-glucose over L-glucose upon starvation, which do not appear to be caused by aberrant hemolymph glycemia or glycogen levels (Figures 4B and S7). These defects were rescued by the expression of a *UAS-Dh44* transgene by the P_{Dh44}-GAL4 driver. Additional support for this was provided by the observation that flies with targeted knockdown of the *Dh44* transcript in these neurons by *Dh44* RNAi were impaired in their ability to develop a preference for D-glucose upon starvation (data not shown).

Flies, like mammals, have two receptors for this Dh44/CRH neuropeptide: Dh44 R1 and Dh44 R2. In *Drosophila*, these receptors are both activated by Dh44 peptide (Hector et al., 2009; Johnson et al., 2003) To determine whether these receptors are necessary for flies to be able to select nutritive sugars, we used *Dh44 R1* and *R2* mutants. These mutants failed to develop a preference for D-glucose over L-glucose in the two-choice assay when starved (Figures 4C and 4E). Moreover, we generated GAL4 lines using the putative promoters for *Dh44 R1* and *Dh44 R2*. Inactivation of Dh44 R1 neurons by expression of *UAS-Kir2.1* or ablation of Dh44 R2 cells by expression of *UAS-Reaper, Hid*, under the control of these GAL4 lines blocked starvation-induced selection of nutritive D-glucose (Figures 4D and 4F). These results indicate that *Dh44 R1* and *Dh44 R2* receptors and their cells are required for the selection of nutritive sugar.

To further our understanding of how *Dh44 R1* and *Dh44 R2* contribute to food choice behavior, we examined the expression patterns of the P_{Dh44R1}-GAL4 and P_{Dh44R2}-GAL4 lines. We found that the P_{Dh44R1}-GAL4 line drives the expression of *UAS-CD8GFP* in approximately ten cells in the fly brain and three to four pairs of cells in the VNC (Figure 4G, inset) These cells arborize extensively within the PI and extend processes along the midline that overlap with the neurites of Dh44 neurons (see Figure 2B) to innervate the dorsal region of the SEZ. The three to four pairs of Dh44 R1 cells arborize their neurites throughout the VNC (see Figure 4G, inset). In contrast, GFP expression was not observed in the brains of flies carrying P_{Dh44R2}-GAL4 and *UAS-CD8GFP*. Instead, it was seen in a large number of cells in the gut that have the characteristic shape of enteroendocrine cells (Figure 4H, inset).

Artificial Activation of Dh44 R1 Neurons Stimulates Rapid PER

We examined flies in which Dh44 R1 neurons are artificially stimulated by expression of NaChBac. In the two-choice assay, these flies chose sweeter L-glucose even when they were starved for extended periods (Figure 5A). This result is similar to the observation obtained in flies carrying P_{Dh44}-GAL4 and *UAS-NaChBac* (Figure 1E) that equally preferred both sugars. However, activation of these receptor neurons appeared to be more effective in relieving the preference for nutritive sugar and caused the flies to select more palatable L-glucose, which is detected by intact, external sugar receptors. These illustrate that Dh44 R1 neurons, like Dh44 neurons, are sufficient for mediating post-ingestive nutrient selection.

Furthermore, we serendipitously observed dramatic increased PER responses, which promote food intake, when Dh44 R1 neurons were artificially activated. We expressed the inducible heat-activated Transient receptor potential A1 cation channel, *UAS-TrpA1* (Parisky et al., 2008), in Dh44 R1 neurons and tested the flies at a temperature, 30°C, which triggers inward currents through the channel. The natural feeding pattern is characterized by the repeated extension and retraction of the proboscis, and opening and closing of the labella at the tip of the proboscis, which is evoked by contact with food. By contrast, flies with activated Dh44 R1 neurons lifted the rostrum out of the head and opened the labella at a high frequency, even in the absence of food (Figure 5B; Movie S2). These Dh44 R1 neurons do not appear to be motor neurons, since axonal projections to labella muscles were not observed. Instead, their processes innervate the dorsal region of the SEZ, where motor neurons reside, send axonal projections to the muscles, and mediate PER responses (Gordon et al., 2008; Manzo et al., 2012).

The Dh44 System Is Necessary and Sufficient for Gut Motility and Excretion

We also observed that artificial activation of Dh44 R1 neurons resulted in a remarkably increased rate of excretion. Individual flies carrying P_{Dh44R1}-GAL4 and *UAS-TrpA1* excreted significantly higher numbers of waste deposit within 10 min and

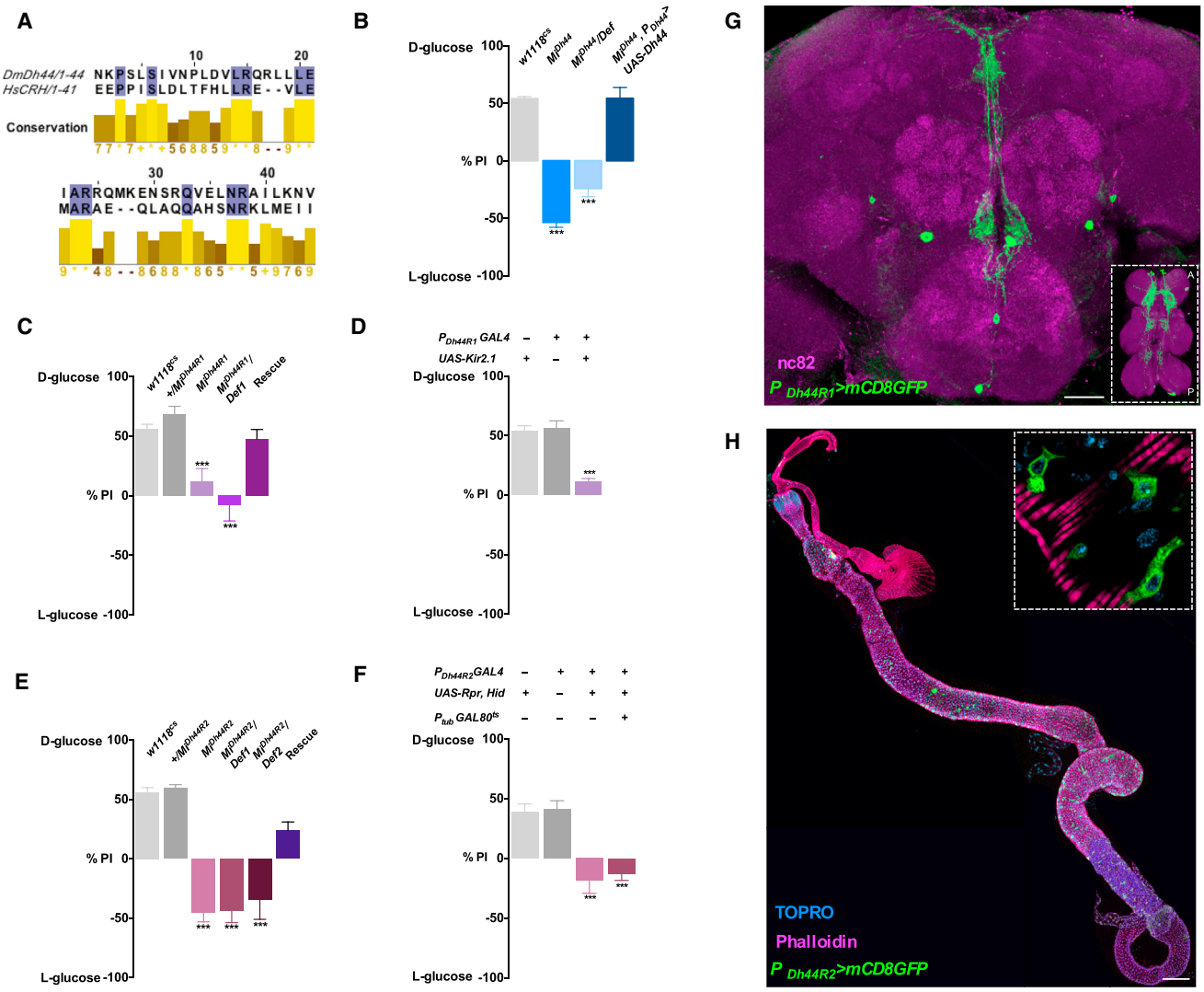

Figure 4. *Dh44*, *Dh44* Receptors, and Their Neurons Are Required for Post-ingestive Nutrient Selection

(A) Alignment of the mature peptide sequence of the human CRH and fly Dh44 neuropeptides using Jalview (Waterhouse et al., 2009). Top: blue indicates identical residues. Bottom: the degree of amino acid conservation demonstrated by the shade of color (bright yellow to dark brown indicates decreasing similarity), the height of histogram bar, and a numerical score (n > 5 high identity, n < 5 low identity, n = + isoleucine/leucine).

(B–F) The food preferences in the two-choice assay (50 mM D-glucose versus 200 mM L-glucose) after 18-hr starvation were measured in the following: (B) *Dh44* mutants (Mi^{Dh44} or Mi^{Dh44}/*Def*; $w1118^{CS}$ and Mi^{Dh44} mutant carrying *UAS-Dh44* under the control of P_{Dh44}-GAL4 were used as controls, n = 3–8); (C) *Dh44R1* mutants (Mi^{Dh44R1} and Mi^{Dh44R1}/*Def*; $w1118^{CS}$, Mi^{Dh44R1}/+ and Mi^{Dh44R1}; P_{Dh44R1} > *UAS-Dh44R1* flies were used as controls, n = 3–9); (D) flies harboring P_{Dh44R1}-GAL4 and *UAS-Kir2.1* (flies carrying each transgene alone were used as controls, n = 4–7); (E) *Dh44R2* mutants (Mi^{Dh44R2}, Mi^{Dh44R2}/*Def1*, and Mi^{Dh44R2}/*Def2*; $w1118^{CS}$, Mi^{Dh44R2}/+ and Mi^{Dh44R2}; P_{Dh44R2} > *UAS-Dh44R2* flies were used as controls, n = 3–9); and (F) flies carrying P_{Dh44R2}-GAL4 and *UAS-Reaper, Hid* and flies carrying P_{Dh44R2}-GAL4, *UAS-Hid*, and $P_{tubulin}$-GAL80^{ts} tested after GAL80^{ts} was inactivated (flies carrying either P_{Dh44R2}-GAL4 or *UAS-Reaper, UAS-Hid* transgene alone were used as controls, n = 3–10). ***p < 0.001 (one-way ANOVA with Tukey post hoc tests).

(G) A z-stack image of the brain of a fly carrying P_{Dh44R1}-GAL4 and *UAS-mCD8GFP* (green) counterstained with the neuropil marker, nc82 (magenta). Scale bar, 50 μm. Inset, the expression of P_{Dh44R1}-*GAL4* > *mCD8GFP* in the VNC. A, anterior; P, posterior.

(H) A z-stack image of the midgut of a fly carrying P_{Dh44R2}-GAL4 and *UAS-mCD8GFP* counterstained with phalloidin (pink) and TOPRO (DNA, cyan). Scale bar, 200 μm. Inset, Magnified image of P_{Dh44R2}-*GAL4* > *mCD8GFP* in a subset of enteroendocrine cells (green) in the midgut.

Error bars indicate SEM.

approximately 5-fold higher numbers of waste deposit within 60 min than the control flies after they were incubated at 30°C (Figure 6A). The number of excreta from a population of 30 flies was 3-fold higher than that from the control flies (Figure 6B). This finding is in accordance with previous reports in which the rapid release of Dh44 into the circulation after meals resulted in increased rates of excretion in other insects (Audsley et al., 1997; Iaboni et al., 1998). Conversely, mutants for *Dh44* and *Dh44* receptors yielded significantly fewer excreta than the control flies (Figure 6C). This result further supports that the rate of

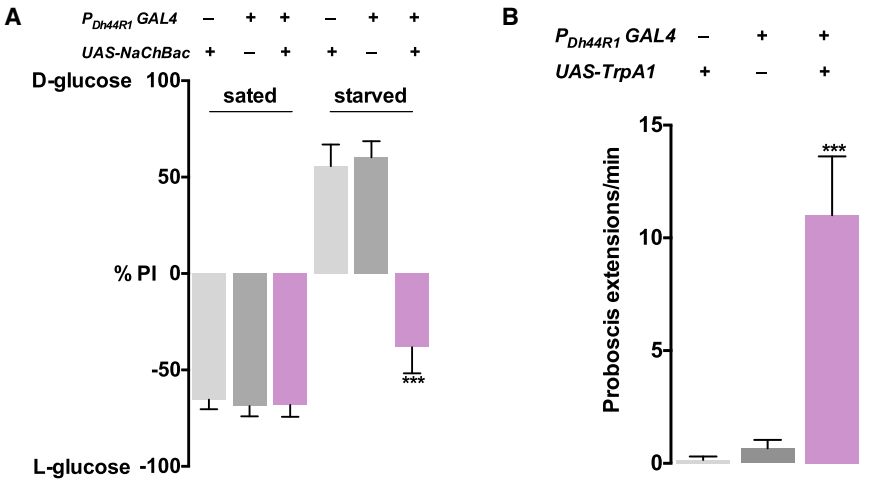

Figure 5. Artificial Activation of Dh44 R1 Neurons Results in Rapid Proboscis Extension, Even in Absence of Food

(A) The food preferences of flies carrying P_{Dh44R1}-GAL4 and *UAS-NaChBac* in the two-choice assay (50 mM D-glucose versus 200 mM L-glucose) after 5-hr (sated) and 18-hr (starved) food deprivation. Flies harboring each transgene alone were used as controls. n = 4–10.

(B) Acute temperature-induced activation of Dh44R1 neurons in flies bearing P_{Dh44R1}-GAL4 and *UAS-TrpA1* at 30°C promotes robust PER responses in the absence of food. Flies carrying each transgene alone were used as controls. n = 12–15. ***p < 0.001 (one-way ANOVA with Tukey post hoc test).

Error bars indicate SEM.

excretion is stimulated by the Dh44 neuropeptide, which is released by the consumption of food containing nutritive sugars.

Corticotropin-releasing hormone (CRH) in mammals was shown to promote gut motility through CRH receptors in the gut (Tache and Perdue, 2004). Therefore, we asked whether Dh44 peptide increases the gut motility. The spontaneous contractions of the dissected fly gut were measured after Dh44 peptide was perfused onto the gut preparation. We found that Dh44, at a concentration previously shown to activate Dh44 receptors (Hector et al., 2009; Johnson et al., 2003), stimulated the gut motility approximately 3-fold, compared to the control saline (Figure 6D; Movies S3 and S4). Conversely, Dh44-induced enhancement of the gut motility was eliminated in *Dh44 R2* mutants (Figure 6E; Movies S5 and S6) and was unaffected in mutants for *Dh44 R1* (data not shown). These results indicate that coordinated activity of the Dh44 microcircuit regulates appropriate gut motility and excretion.

Flies Rapidly Detect the Nutritional Value of Sugar

Next, we sought to understand how roaming flies in the two-choice arena readily distinguish a nutritive sugar from a nonnutritive sugar. We investigated the possibility that flies might be capable of detecting the nutritional value of sugars within few minutes of ingesting food. To this end, we carried out a time course study; the preference of flies was scored every few minutes after they began to ingest either D-glucose or L-glucose in the two-choice assay. We found it intriguing that starved wild-type flies initially selected sweeter L-glucose but started to choose nutritive D-glucose within 5 min (Figure 7A). By contrast, starved *Dh44* mutants chose sweeter L-glucose and failed to develop a preference for D-glucose. The finding suggests that the nutritional value of D-glucose is detected in a relatively fast timescale. Consistent with this, a significant increase in hemolymph glycemia was observed within a few minutes of ingesting food (Figures 7B and 7C). The rapid detection enables immediate stimulation of innate behavioral programs that lead to the continuation of D-glucose ingestion through increased PER responses and the activation of food processing in the gut (Figure 7D).

DISCUSSION

The Dh44 System Directs the Detection and Consumption of Nutritive Sugars

We have identified the molecular and cellular nature of a sensor in the brain that detects the nutritional value of sugar through direct activation by nutritive sugars. Dh44 neurons are activated specifically by nutritive D-glucose, D-trehalose, and D-fructose, which are normally found in the hemolymph, and are not activated by nonnutritive sugars or sugars that are not found in the hemolymph. Sugar-induced activation of these six central neurons resulted in secretion of the Dh44 neuropeptide, which transmits a signal to Dh44 R1 and R2 cells. Flies in which the expression of *Dh44* or *Dh44* receptors is disrupted or the function of Dh44 receptor cells is inactivated failed to develop a preference for nutritive sugar.

Insight into the contribution of the Dh44 downstream effectors to the selection of nutritive sugars was gained in the TrpA1-mediated activation experiment. We made the surprising observation that artificial activation of Dh44 R1 neurons rapidly induced PER responses, even in the absence of food. Stimulation of Dh44 R1 neurons also caused the flies to excrete large amounts of waste deposits; conversely, inactivation of the Dh44 circuit resulted in deceleration of gut motility and excretion. Together, we propose that the Dh44 system not only mediates detection of the nutritional content of sugar but also coordinates the ingestion and digestion of sugar by promoting proboscis extension and by promoting gut motility and excretion through a positive feedback loop (see Figure 7D).

Dh44 Neurons: The Post-ingestive Nutrient Sensor

Two possible mechanisms could explain how flies can make appropriate food choices in the two-choice assay. One mechanism is regulated by a post-ingestive nutrient sensor that detects the nutritional value of D-glucose through direct activation during the postprandial rise in hemolymph glycemia. Another mechanism is mediated by a prescriptive "hunger" sensor that monitors the status of the internal energy reservoir and promotes consumption of nutritive D-glucose after periods of starvation.

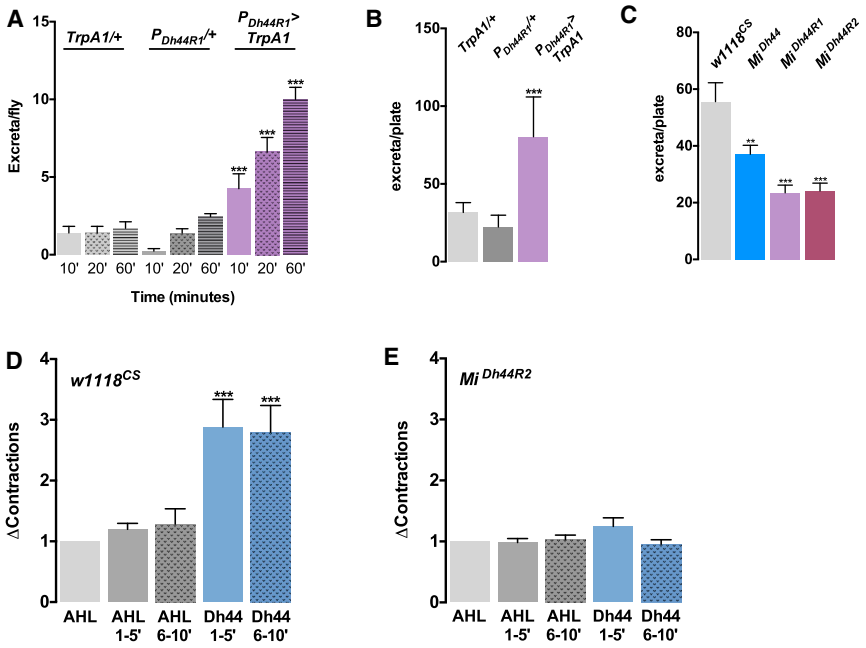

Figure 6. The Dh44 System Is Necessary and Sufficient for Gut Motility and Excretion

(A) Number of excreta in individual P_{Dh44R1}-*GAL4* > *TrpA1* flies starved for 18 hr was measured at different time points at 30°C. Flies carrying each transgene alone were used as controls. n = 13–15. ***p < 0.001.

(B and C) Number of excreta in a population of 30 flies. (B) Flies carrying P_{Dh44R1}-GAL4 and *UAS-TrpA1* and flies carrying each transgene alone were tested at 30°C. n = 3. ***p < 0.001. (C) *Dh44*, *Dh44R1*, and *Dh44R2* mutants and $w1118^{CS}$ control flies. n = 5–17. **p < 0.01; ***p < 0.001.

(D and E) Gut propulsivity of (D) $w1118^{CS}$ or (E) Mi^{Dh44R2} mutant in response to the control AHL saline (gray bars) and AHL containing Dh44 peptide (10^{-6} μM) (blue bars). AHL 1-5′ and Dh44 1-5′ refer to minutes 1–5 incubated in AHL and AHL containing Dh44 peptide; AHL 6-10′ and Dh44 6-10′ refers to minutes 6–10 incubated in AHL and AHL containing Dh44 peptide, respectively. Δcontractions (on the y axis) were calculated by normalizing the number of contractions in AHL containing Dh44 peptide over those in the control AHL. n = 9–17 guts. ***p < 0.001 (one-way ANOVA with Tukey post hoc test).

Error bars indicate SEM.

Several lines of evidence suggest that Dh44 neurons function as a post-ingestive nutrient sensor. First, Dh44 neurons are activated specifically by nutritive sugars and not by nonnutritive sugars. Second, Dh44 neurons are capable of directly sensing the nutritional value of sugar, as sugar-induced calcium responses were not eliminated in fly brains treated with TTX, a sodium channel blocker that abolishes synaptic transmission. Third, flies with Hex-C knocked down in these Dh44 neurons had impaired responses to nutritive sugar. Fourth, artificial activation of Dh44 neurons or Dh44 R1 neurons significantly reduced the preference for nutritive sugars, even when the flies were starved, because activation of the putative nutrient-sensing pathway was sufficient to communicate the reward of nutrient. Therefore, starved flies carrying P_{Dh44}-GAL4 and *UAS-NachBac* equally preferred D- and L-glucose. This is in contrast to another population of central neurons identified from a screen that functions as a prescriptive hunger sensor. When these neurons were artificially activated, the flies chose a nutritive sugar over a nonnutritive sugar, even when they were sated (M.D. and G.S.B.S., unpublished data). Finally, either activation or inactivation of Dh44 neurons did not alter the amount of food consumption. This is distinct from manipulating the prescriptive hunger sensor that had substantial effects on the amount of food intake (M.D. and G.S.B.S., unpublished data). These support the assertion that the glucose-sensing Dh44 neurons guide flies to recognize the nutritional value of sugar by directly monitoring circulating sugar levels and utilizing sugar molecules.

Flies Detect the Nutritive Value of Sugar in a Fast Timescale

The means by which flies distinguish D-glucose from L-glucose are not understood. It was proposed that flies roaming in the two-choice arena find D-glucose by associating a spatial cue, the location of the D-glucose containing agar, with the nutritional content of D-glucose. The observation that these flies are capable of selecting D-glucose even in the dark (Dus et al., 2013), however, suggests that spatial conditioning is unlikely to be involved in post-ingestive food choice behavior. Rather, the detection of nutritive D-glucose appears to be mediated by interoceptive chemosensory neurons that elicits innate behavioral responses, similar to the sweet-evoked chemosensory responses mediated by external sweet receptors. Upon activation by a nutritive sugar, the interoceptive chemosensory neurons stimulate a constellation of behavioral sub-programs that result in a positive feedback for the selection and consumption of nutritive sugar.

Consistent with this hypothesis, the post-ingestive nutrient sensor functions in a fast timescale. Calcium imaging of dissected ex vivo brain preparations, which may not reflect the in vivo context in which ingested foods pass through the digestive tract, showed that the activity of Dh44 neurons is rapidly stimulated when exposed to nutritive sugar. Furthermore, hemolymph glycemia significantly increases as soon as flies start to ingest sugars (Figure 7B). The rise of hemolymph glycemia would readily stimulate the activity of Dh44 neurons, which are located adjacent to insulin-producing cells (IPCs) in the PI that also respond to sugar. Finally, the time-course experiment demonstrated that flies that begin to feed in the two-choice assay are capable of responding correctly to nutritive D-glucose within 5 min. These results support the view that flies recognize the nutritional content of D-glucose rapidly after ingestion.

Glucose-Sensitive Neurons in the Brain

It has been 5 decades since the glucostatic hypothesis was proposed, yet it is still uncertain whether glucose-sensing neurons in the brain have a role in food intake or nutrient selection. Mice that

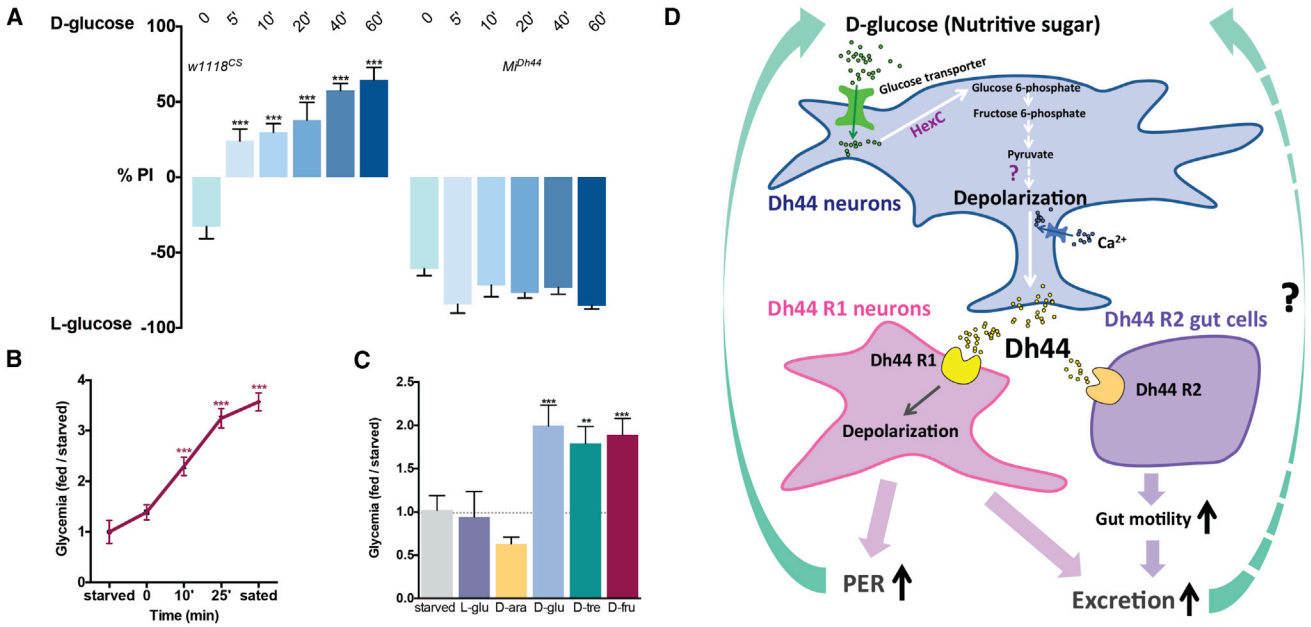

Figure 7. Flies Promptly Detect the Nutritional Value of Sugar

(A) The food preference in the two-choice assay (50 mM D-glucose versus 200 mM L-glucose), measured at different time points (x axis) in $w1118^{CS}$ and Mi^{Dh44} mutant flies. Time point 0′ is the time at which the majority of flies started to feed. n = 4–10, ***p < 0.001 (one-way ANOVA with Dunnet post hoc test).

(B) Measurement of circulating glucose and trehalose levels in 18-hr-starved male $w1118^{CS}$ flies that were fed with 100 mM D-glucose. Their hemolymph was collected at different time points (x axis) for each measurement. The data were normalized to hemolymph glycemia in 18-hr-starved flies. 0′ refers to the time at which the majority of flies began to eat. n = 6–11. ***p < 0.001.

(C) Measurement of circulating glucose and trehalose levels in 18-hr-starved male flies that were fed with different sugars at 100-mM concentrations for 15 min. The data were normalized to hemolymph glycemia in 18-hr-starved flies. n = 7–14. **p < 0.01; ***p < 0.001, with respect to the starved flies.

(D) The dynamics of Dh44-mediated sugar sensing. Ingestion of nutritive sugar leads to the activation of Dh44 neurons, which results in the release of Dh44 neuropeptide. Dh44 (CRH) subsequently acts on different sites to activate PER responses and to stimulate the gut motility and excretion through a positive feedback loop.

Error bars indicate SEM.

lack a critical signal transducer, either AMPK or K_{ATP} channel, in their glucose-sensing neurons and, thus, lack the ability to sense extracellular glucose display essentially normal feeding behavior (Claret et al., 2007; Parton et al., 2007). A study in rats also showed a lack of any causal relationship between blood and hypothalamic glucose levels and daily meal initiations (Dunn-Meynell et al., 2009). Recently, hypothalamic glucose-sensing melanin-concentrating hormone (MCH) neurons were shown to respond to and communicate the nutritional value and reward of sugar, but it was not clear whether the glucose excitability of these MCH neurons mediated the behavioral response (Domingos et al., 2013; Kong et al., 2010). However, central administration of 2-deoxyglucose or insulin-induced hypoglycemia does elicit food intake (Dunn-Meynell et al., 2009; Miselis and Epstein, 1975). It was speculated that extremely low brain glucose levels trigger food intake through the action of unidentified hypothalamic glucose-sensing neurons, which may protect against the dangers of hypoglycemia in mammals (Routh, 2010).

Our study in *Drosophila* showed that the glucose excitability of Dh44 neurons mediates starvation-induced selection of nutritive sugars, which depends on the sugar entry and the function of Hex-C to convert the glucose into its metabolic product, glucose-6-phosphate. This step in the glucose metabolic pathway appears to be critical for stimulating the neuronal activity in Dh44 neurons and responding to the nutritional value of sugar. It is noteworthy that Hex-C mRNA is expressed in few regions, including the brain, whereas another fly hexokinase, hexokinase A (Hex-A), is expressed in nearly all tissues in the fly. The intracellular glucose metabolism initiated by Hex-C, possibly through the generation of sugar metabolites, is important for detecting the nutritional value of D-glucose that elicits innate preference behavior.

Dh44: A *Drosophila* Homolog of CRH

Since it was discovered 25 years ago, CRH has been characterized as a hypothalamic hormone that communicates stress responses. CRH also plays a significant role in the regulation of energy balance, but the exact nature of its role is controversial. CRH appears to have an anorectic effect in rodents (Richard et al., 2002) but has an opposite effect in humans when calorie intake is stimulated by an infusion of CRH (George et al., 2010). The homology between *Drosophila* Dh44 and mammalian CRH is approximately 30% and between *Drosophila* and mammalian receptors is approximately 40%; this suggests that the function of these two systems is conserved. Indeed, mammalian CRH, which is similar in function to *Drosophila* Dh44, is required for the regulation of gastric and colonic movements; notably, CRH administration was shown to stimulate defecation in rodents (Tache and Perdue, 2004). Furthermore, CRH mediates glucose

homeostasis by regulating hypoglycemia-induced counterregulation (CRR) (McCrimmon et al., 2006). CRR triggers a number of responses that limit glucose utilization, promote endogenous glucose production, and lead the animal to seek food. It has been suggested that the function of glucose-sensing neurons is to generate neuroendocrine stress responses to the hypoglycemic challenge, but the identity of these neurons is unknown. It would be interesting to investigate the possibility that CRH neurons, which are expressed in the hypothalamus, are glucose-sensing neurons and capable of mediating starvation-induced behavioral responses to the nutritional value of sugar in mammals. A stress-responsive CRH system might be co-opted to allow animals to respond to the stress of starvation.

EXPERIMENTAL PROCEDURES

Fly Strains

Flies were grown in standard cornmeal-molasses medium at low density at 25°C. *w1118* flies backcrossed to *Canton-S* (*CS*) ten generations (referred as $w1118^{CS}$), kindly provided to us by Dr. Anne Simon, were used as control. *Dh44* (Flybase: CG8348, #25345, *w1118; Mi{ET1}Dh44*$^{[MB07006]}$), *Dh44R1* (Flybase: CG8422, #23517, *w1118; Mi{ET1}Dh44-R1*$^{[MB03192]}$), *Dh44R2* (Flybase: CG12370, *w1118; Mi{ET1}Dh44-R2*$^{[MB10503]}$, #29129) mutants, deficiencies (Flybase: #26552, #7731, # 27929, #26388), and *Dh44* and *hexokinases* RNAi lines (Flybase: #35338-Hex-C, #47331-Hex-T2, #46574-Hex-T1, #35155-Hex-A) were obtained from the Bloomington *Drosophila* Stock Center at Indiana University. *UAS-Kir2.1*, *tubulin-GAL80*^{ts}, and *UAS-TNT* were from Dr. David Anderson (California Institute of Technology); *UAS-NaChBac* was from Dr. Justin Blau (New York University [NYU]); *UAS-Trp1* was from Dr. Paul Garrity (Brandeis University); *UAS-GCaMP3.0* and *UAS-GCaMP6.0* were from Dr. Loren Looger and Dr. Jayaraman (Janelia Farm Research Campus); *UAS-mCD8GFP*, *UAS-Dscam-GFP, UAS-mko*, and *UAS-Synaptotagmin-GFP* were from Dr. Ann-Shyn Chiang (National Tsing Hua University); and *UAS-Reaper, UAS-Hid* was from Dr. Don Ryoo (New York University).

Transgenic Lines

The P_{Dh44}-*GAL4* line was generated by cloning an 800-bp region upstream of the *Dh44* promoter into pCasper4-AUG-GAL4X. The P_{Dh44R1}-*GAL4* and P_{Dh44R2}-*GAL4* lines were generated in the same way by cloning the ~1-kb fragment upstream of the *Dh44R1* and *Dh44R2* open reading frame (ORF) into pCasper4-AUG-GAL4X. The *UAS-Dh44*, *UAS-Dh44R1*, and *UAS-Dh44R2* rescue constructs were cloned by RT-PCR using total fly RNA and were subsequently subcloned into a pUAST:attb vector. Transgenic flies were generated by Bestgene.

Two-Choice Assay

The two-choice preference assay was conducted as previously described (Dus et al., 2011). Briefly, approximately 40 male flies 4 to 8 days old were food deprived in an empty vial with Kimwipe wetted with 2 ml of MilliQ water for 5 or 18 hr and then given a choice between two sugars, each color coded with a tasteless food dye, for 2 hr. Food preference was scored as percent preference index (% PI), shown below:

$$\%\ \mathrm{PI} = \frac{(\#\ \text{flies ate food1} + 0.5^{*}\#\ \text{flies ate both}) - (\#\ \text{flies ate food2} + 0.5^{*}\#\ \text{flies ate both})}{(\text{total}\ \#\ \text{flies ate})}.$$

All sugars, except for L-glucose (Carbosynth), were from Sigma.

Immunofluorescence

Immunohistochemistry of the brains was conducted according to the protocol of Chiang et al., (2011). Gut immunostaining was performed as in Dus et al., (2013), with an extra step in which flies were fed agar-based food for 2 days to decrease background. Antibodies used were as follows: mouse anti-nc82 (1:50; Developmental Studies Hybridoma Bank), rabbit anti-GFP immunoglobulin G (IgG) (1:500; Invitrogen), goat anti-mouse-biotin (1:200), and rabbit anti-Dh44 (1:500) (Zitnan et al., 1993). Secondary antibodies were Alexa Fluor 647-Strepavidin (1:500, Invitrogen) and Alexa Fluor 555 goat anti-rabbit IgG (1:500, Invitrogen); TO-PRO3 (1:500; Invitrogen) was used for DNA labeling; Alexa 555-Phalloidin (1:200, Invitrogen) was used for gut immunostaining. Images were acquired with a Zeiss LSM 510 or Zeiss LSM 700 confocal microscope with 1- to 2-μm optical sections at a 1,024 × 1,024 or 512 × 512 resolution.

Calcium Imaging

Adult fly brains were dissected with sugar-free adult hemolymph-like solution (AHL) and immobilized with fine tungsten pins on a Sylgard-based perfusion chamber. During perfusion, appropriate concentrations of sucrose were added to sugar-free AHL before and after the stimulus to balance the difference in osmolarity. Each brain was recorded for 500 frames in total (512 × 512 pixels; one frame per 5 s); the first 100 frames were recorded before each stimulus was presented, the next 200 frames were recorded during the exposure to sugar/drug, and the following 200 frames were recorded during washout. Solutions in the perfusion chamber were operated by pinch valves, which were controlled by a ValveBank controller (AutoMate Scientific). Changes in the fluorescence intensity were recorded with a Prairie two-photon microscope with a 40× water immersion lens (Olympus). Pseudo-color images and image analyses were performed using ImageJ. Note that a single outliner was removed from quantifying the glucose + phlorizin dataset (1/9).

Measurement of Intracellular Dh44

The brains of 18-hr-starved flies were rapidly dissected in sugar-free AHL, incubated in either AHL saline (108 mM NaCl, 8.2 mM $MgCl_2$, 4 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2 mM $CaCl_2$, 5 mM KCl, 5 mM HEPES + 80 mM sucrose to balance the osmolarity) or AHL+ 80 mM sugars for 30 min, and then fixed and stained with anti-Dh44 antibody as per the immunofluorescence protocol described earlier. Image acquisition was conducted using a Zeiss LSM 510 confocal microscope with a fixed gain setting between samples. ImageJ software was used to quantify the fluorescence intensity per cell.

Measurement of Gut Motility

The guts from 18-hr-starved flies were dissected in AHL without disrupting attached tissues and without removing the head, muscles, or fat (Talsma et al., 2012). The exposed gut was pinned onto a Sylgard plate, with fine tungsten pins through the proboscis and a small piece of cuticle attached to the end of the gut, and bathed in 13 μl of AHL. Each gut was imaged with a Zeiss high-speed camera (two frames per second) connected to a stereomicroscope with 0.6× magnification. After 5 min in AHL, the solution was removed by capillary action and replaced with 13 μl AHL containing 10^{-6} μM Dh44 peptide or AHL alone. Video acquisition rapidly restarted for 10 min. Each video was processed with the Zeiss AxioPlan 4.8 software and converted into a MP4 file with seven frames per second. Quantification of gut contraction was conducted by visually counting for 1 min after an addition of the solution to avoid diffusion artifacts: In Figure 6, AHL 1-5′ and Dh44 1-5′ refer to incubation 4 min long (1–5 min); AHL 6-10′ and Dh44 6-10′ refer to incubation 4 min long (6–10 min). The real-time video was accelerated four times (1 min in real time equates to 15 s in the video). Contractions of each gut were normalized to the number of contractions in the initial AHL solution.

PER

A fly starved for 18 hr was gently trapped into a chopped p200 pipette tip to expose the head and forelegs to stimuli. Each tip was placed perpendicularly onto a slide covered with clay and positioned at the bottom of a stereomicroscope in a room heated to 30°C. After 5 min, each fly was observed through the objective of the microscope, and their PER responses were counted. To obtain a video, flies were gently trapped into a glass Pasteur pipette with a small cotton plug and transferred to a 30°C heated room for 5 min, where the footage was captured using a Zeiss high-speed camera and stereomicroscope at two frames per second.

Excretion Assay

Single-Fly Assay

A starved single male fly was gently introduced into a glass Pasteur pipette sealed with a small cotton plug and ~5 μl water to prevent desiccation and was immediately transferred to a 30°C heated room. The number of excreta (visible on the glass wall) was counted after 10, 20, and 60 minutes.

Population Assays

Thirty male flies previously fed food + 0.1% blue dye (erioglaucine) for 3 days were introduced into a 5-cm plastic Petri dish containing filter paper. These flies were either kept in room temperature or immediately transferred to a 30°C heated room for 60 min. The number of excreta on the filter paper was quantified visually with a stereomicroscope.

Measurement of Hemolymph Glycemia

Hemolymph glycemia was measured as previously described (Dus et al., 2011).

Statistics

GraphPad Prism software was used for all graphs and statistical analyses. A Student's t test or one-way ANOVA was used.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.05.032.

AUTHOR CONTRIBUTIONS

G.S.B.S., M.D., and J.S.-Y.L. designed, analyzed, and interpreted the experiments. G.S.B.S. wrote the manuscript with M.D. and other authors. M.D. performed the screen; all the behavioral experiments; immunostaining; and excretion, glycemia, and glycogen measurements. J.S.-Y.L. performed all the calcium-imaging experiments and immunostaining. K.M.G. made the P_{Dh44R1}-GAL4 and P_{Dh44R2}-GAL4 drivers and rescue constructs. S.M. showed DH44 neuronal projections in the gut. T.D.T. and A.C.H. constructed the neuropeptide GAL4 lines. E.G. and C.M.J. helped with the excretion measurement. G.S.B.S. conceived and supervised the project.

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